http://dx.doi.org/10.5012/bkcs.2013.34.7.2081

Quantitative Determination of Marker Compounds and Pattern Recognition Analysis for Quality Control of Alismatis Rhizoma by HPLC

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Received January 3, 2013, Accepted April 16, 2013

A quantitative method for determining levels of three bioactive compounds based on pattern recognition was developed and fully validated for the quality control of Alismatis Rhizoma (AR) by HPLC. Separation conditions were optimised using an Optimapak C_{18} column (250 mm × 4.6 mm, 5 μm) with a mobile phase of acetonitrile and 0.1% aqueous phosphoric acid and detection wavelengths of 205 and 245 nm. Method validation yielded acceptable linearity ($r^2 > 0.9998$) and percent recovery (98.06%-101.71%). Limits of detection ranged from 0.08 to 0.15 μg/mL. Levels of the three bioactive compounds, alisol C acetate, alisol B, and alisol B acetate, in AR were 0.07-0.45, 0.38-10.32, and 1.13-8.59 mg/g dried weight, respectively. Pattern analyses based on these three compounds were able to differentiate Chinese and Korean samples accurately. The results demonstrate that alisol B and its acetate may be used as marker compounds for AR quality and can be regulated to no less than 0.36 and 1.29 mg/g of dried sample, respectively. The method described here is suitable for quantitative analyses and quality control of multiple components in AR.

Key Words : Alismatis Rhizoma, Marker compound, Alisol B, Quality control, Pattern analysis

Introduction

Alismatis Rhizoma (AR) is a well-known traditional medicine prepared from the tuber of *Alisma orientale* Juzepzuk (Alismataceae) after removing the periderm.[1] *A. orientale* is an aqueous plant of the Alismataceae family, which contains approximately 90 species and 11 genera worldwide but is distributed primarily across East Asia.[2] In traditional oriental medicine, *A. orientale* has been used as a diuretic agent.[3] AR is also a component of several oriental herbal preparations such as Taeg-Sa-San and O-Ryeong-San and is believed to lower cholesterol, act as a diuretic, and to have anti-allergic, anti-inflammatory, and antibacterial properties. Chemical and pharmacological investigations of AR have resulted in the discovery of several bioactive components including as protostane-type triterpenes,[4,5] guaiane-type sesquiterpenes,[6] and kaurane-type diterpenes.[7] These compounds have been credited with the various pharmacological activities of this herb including diuretic,[6,8] antihypertensive,[7] anticomplement,[9,10] cytotoxic,[11] iNOS inhibitory,[12] antiallergic,[13] and antitumour[14] effects. The AR currently available in the marketplace mainly comes from Korea and China; because it contains varying contents of bioactive substances, the therapeutic effects of AR are uncertain. Quality control of AR is important to guarantee its clinical efficacy and safety.

Traditional approaches to quality control select one or a few active compounds for identification and quality evaluation. In contrast, fingerprint approaches use the internal relationships of compounds to reveal the chemical pattern of herbal drugs, and can provide more information than traditional methods. Chromatographic fingerprint or pattern recognition analysis, together with quantitative analysis, has been recommended as a suitable method for assessing the quality of herbal drugs.[15-19] The multi-peak information content of a chromatogram can be analysed using various approaches such as fingerprint, classification, differentiation, or a combination of quantitative and pattern analysis.[19-23] To date, there is no mention of marker compounds in Korean, Chinese, or Japanese pharmacopoeias for the quality control of AR. Our previous research revealed that alisol B acetate was an appropriate and quantifiable marker compound for the quality control of AR.[24] However, alisol B also appears in a relative high content in AR, especially in Chinese AR samples. This indicates that a multi-component analysis is needed to ensure effective quality control of AR.

Several analytical methods employing high-performance liquid chromatographic (HPLC) separation and ultraviolet (UV) absorbance detection have been developed for the analysis of AR.[24-27] However, these studies have focused only on quantitative analyses of selected marker compounds. As noted above, this is not the most appropriate approach for developing quality control protocols for multicomponent herbal drugs. The present study describes a simple, sensitive, and precise reverse-phase HPLC/UV method for quantifying three marker compounds: alisol C acetate (**1**), alisol B (**2**), and alisol B acetate (**3**), along with a pattern recognition analysis to be used for the quality control of AR. For pattern recognition with a multivariate statistical analysis, the R

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program (http://www.r-project.org) was used to analyse 86 samples of AR from Korea and China. The results verified that this method is suitable for quality control of AR.

Experimental Section

Instrumentation and Reagents. The HPLC system consisted of an LC-20AD pump, SPD-20A UV/Vis detector, SIL-20A autosampler, and a CTO-20A column oven (Shimadzu, Japan). HPLC grade reagents, acetonitrile, and methanol were purchased from Burdick & Jackson (Morristown, NJ, USA). The internal standard (IS), propyl paraben, was purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals were of analytical grade unless otherwise noted. Double-distilled water was prepared using an ultrafiltration system (Shinhan Scientific, Korea).

Standard Chemicals and Plant Materials. All standard compounds were provided by Prof. Eun Ran Woo, College of Pharmacy, Chosun University. The standard compounds, shown in Figure 1, were unambiguously identified as alisol C acetate (MW 528.73), alisol B (MW 472.70), and alisol B acetate (MW 514.74) by structural analyses using nuclear magnetic resonance and mass spectrometric data.[4,28,29] The purity of these standards was estimated at 96.66% for alisol C acetate, 96.51% for alisol B, and 97.26% for alisol B acetate based on HPLC and LC-MS analyses. Analytical samples of the herbal drug Alismatis rhizoma were collected from Korea (53 samples) and China (33 samples) in 2011. The collected samples were identified by Prof. Jae Hyun Lee, College of Oriental Medicine, Dongguk University, and voucher specimens were deposited in the herbarium at the College of Pharmacy, Chungnam National University.

Standard Solution and Calibration. A stock solution (1 mg/mL) of alisol B, alisol B acetate, alisol C acetate, and propyl paraben (IS) was prepared in methanol and kept below 4 °C. Standard solutions were prepared by serial dilution of the stock solution to working ranges of these substances with methanol.

Sample Preparation. Dried tuber powder was used for each extraction. Approximately 200 mg of moderately coarse AR powder was accurately weighed and placed in a 10-mL volumetric flask, along with 50 μL of 1 mg/mL IS, and diluted to the mark with 100% methanol. The filled flask was weighed and the solution was ultrasonically extracted for 60 min. The solution was cooled and weighed again. Any losses in weight were compensated with additional methanol. The sample mixture was filtered through a 0.22-μm membrane filter and a 10-μL sample of aliquot of the filtrate was subjected to HPLC analysis.

Chromatographic Conditions. HPLC analysis was conducted using an Optimapak C_{18} column (4.6 mm × 250 mm, 5 μm; RStech Corp., Daejeon, Korea) at a controlled temperature of 40 °C. The mobile phase consisted of a gradient elution with solvent A (0.1% phosphoric acid in water) and solvent B (acetonitrile). The proportion of solvent B was increased from 50% to 100% over 35 min, then maintained at 100% for another 10 min. The mobile phase flow rate was 0.8 mL/min. Optical absorption of the eluate was monitored at 205 nm (alisol B and its acetate) and 245 nm (alisol C acetate). Data were processed using LC Solution Pro software (Shimadzu).

Optimisation of Extraction Efficiency of Main Compounds. Extraction solvent (50%, 75%, and 100% ethanol, and 50%, 75%, and 100% methanol), extraction time (10, 30, 60, 90, and 120 min), and extraction method (shaking, refluxing, and ultrasonication) were evaluated by measuring the amount of bioactive components obtained under each set of conditions.

Validation. Calibration curves were fit to the equation $y = ax + b$ using a linear regression in which y and x were the HPLC analyte peak area relative to that of the IS and compound concentration, respectively. To determine the limits of detection (LOD) and limits of quantitation (LOQ), standard stock solutions were serially diluted in methanol and injected into the HPLC system for analysis. LOD was defined as the concentration at which the least intense detectable peak in the chromatogram had a signal-to-noise (S/N) ratio of three. LOQ was defined as the lowest quantitative level with an S/N ratio of 10. Intra-day precision and accuracy were examined by analysing the standard solutions five times within a single day. Inter-day precision and accuracy were determined by measuring samples on five different days. Recovery tests were performed to evaluate the accuracy of the method by spiking known quantities of mixed standards into samples with a known quantity of each standard. The spiked samples were then extracted and analysed immediately. The added standards were prepared at three different concentrations and each concentration was analysed five times. Stability of the standard methanol solutions was evaluated at 4 °C and 25 °C after aging for 30 and 10 days, respectively.

Pattern Recognition Analysis. Pattern recognition was performed using three different models: linear discriminant analysis (LDA), partial least-squares discriminant analysis (PLS-DA), and k-nearest neighbor (KNN). These analyses were used to differentiate AR samples from Korea and China.

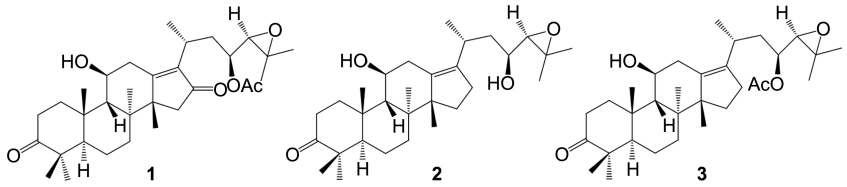

Figure 1. Chemical structures of marker compounds in Alismatis Rhizoma: (**1**) alisol C acetate, (**2**) alisol B, and (**3**) alisol B acetate.

Results and Discussion

Optimisation of Extraction Methods. Six extracting solvents were evaluated with regard to marker compound extraction efficiency. When samples were extracted with 100% methanol, the extracted amounts of marker compounds were greater than those with other solvents. Therefore, 100% methanol was employed as the extraction solvent. While the extraction efficiency of ultrasonication was similar to that of refluxing, the former was chosen for its convenience. All marker compounds were sufficiently extracted after 60 min of room-temperature ultrasonication in 100% methanol.

Optimisation of Chromatographic Conditions. HPLC conditions were optimised to obtain the shortest possible run time while maintaining the baseline resolution of adjacent peaks. Eluents with various acidic modifiers (acetic acid, formic acid, and phosphoric acid) at different concentrations (0.05%, 0.10%, and 0.50%) were evaluated. The addition of 0.1% phosphoric acid to the mobile phase resulted in a good resolution and satisfactory peak symmetry and shape. Various gradient elutions were evaluated with the composition exhibiting the best performance, as described in the Experiments section. Absorbance of the eluate at 205 nm yielded the highest S/N ratio for alisol B and its acetate, while 245 nm was best for alisol C acetate. The highest chromatographic peak resolution with minimal peak tailing was observed at 40 °C. Figure 2 presents typical chromatograms of samples and standard mixtures; note that all target compounds and the internal standard were completely separated within 40 min. The chromatographic peaks of the analytes in sample solutions were identified by comparing their retention times with those of the reference standards and further confirmed by spiking samples with the reference

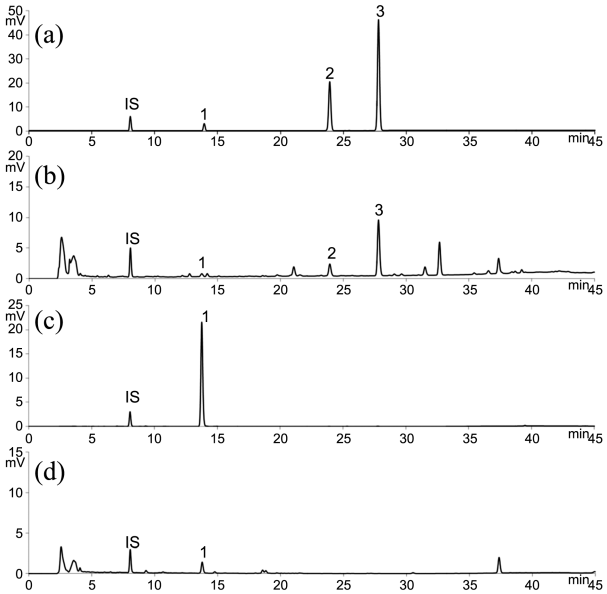

Figure 2. HPLC chromatograms of a standard mixture (a, c) (alisol C acetate 37.5 μg/mL, alisol B 50 μg/mL, alisol B acetate 150 μg/mL) and a Korean Alismatis Rhizoma sample (b, d) (100 mg/mL) detected at 205 nm (a, b) and 245 nm (c, d). Peak (**1**) alisol C acetate, (**2**) alisol B, and (**3**) alisol B acetate.

Table 1. Calibration curves, linear ranges, LODs and LOQs for marker compounds

Compounds	Linear range (μg/mL)	Slope	Intercept	Correlation coefficient (r^2)	LOD (μg/mL)	LOQ (μg/mL)
Alisol C acetate	0.45-150.0	0.0075	0.0020	0.9999	0.15	0.45
Alisol B	0.4-200.0	0.0414	0.0198	0.9999	0.08	0.25
Alisol B acetate	1.2-600.0	0.0295	0.0510	0.9998	0.11	0.35

Table 2. Precision and accuracy of analytical method for standard compounds (n=5)

Compounds	Nominal conc. (μg/mL)	Intra-day			Inter-day		
		Conc. found (μg/mL)	Accuracy (%)	Precision (%)	Conc. found (μg/mL)	Accuracy (%)	Precision (%)
Alisol C acetate	0.45	0.43±0.03	95.12	5.82	0.43±0.03	94.86	6.21
	1.50	1.50±0.02	99.67	1.42	1.50±0.02	99.94	1.54
	7.50	7.49±0.02	99.9	0.31	7.49±0.03	99.83	0.35
	37.50	37.57±0.02	100.19	0.06	37.57±0.09	100.18	0.24
Alisol B	0.25	0.24±0.01	95.41	4.27	0.24±0.01	95.23	4.18
	2.00	2.04±0.01	101.75	0.42	2.02±0.01	101.95	0.51
	10.00	10.07±0.06	100.71	0.63	10.11±0.08	101.75	0.42
	50.00	50.02±0.41	100.04	0.82	50.08±0.51	100.17	1.01
Alisol B acetate	0.35	0.34±0.01	97.35	3.45	0.34±0.02	97.21	4.39
	6.00	6.01±0.06	100.22	1.00	6.04±0.07	100.62	1.09
	30.00	30.44±0.07	101.46	0.23	30.28±0.26	100.92	0.86
	150.00	150.10±0.22	100.06	0.14	150.27±0.43	100.18	0.29

Concentrations are presented as mean ± SD (n=5)

Table 3. Recovery of marker compounds through standard addition (n=5)

Compounds	Added conc. (μg/mL)	Cal. conc. (μg/mL)	Recovery (%)	RSD (%)
Alisol C acetate	0.0	8.15	-	-
	7.5	15.76	101.51	1.86
	15.0	23.14	99.92	0.35
	30.0	38.16	100.05	0.33
Alisol B	0.0	8.83	-	-
	10.0	19.00	101.71	0.39
	20.0	28.44	98.06	0.86
	40.0	48.64	99.51	0.46
Alisol B acetate	0.0	56.54	-	-
	30.0	86.98	101.44	0.61
	60.0	116.28	99.57	0.15
	120.0	176.04	99.58	0.54

compounds in addition to LC-MS analyses.

Validation. Coefficients of correlation (r^2) for three standard calibration points, determined by least-squares analyses, were greater than 0.9997. This result indicates a high degree of linearity between the peak area ratio and compound concentration (see Table 1). LODs and LOQs were at trace levels. Precision and accuracy were determined by multiple analyses (n = 5) of quality control samples prepared at low, medium, and high concentrations spanning the calibration range. As shown in Table 2, the intra- and inter-day precision for the three components ranged from 0.06-5.82% and 0.24-6.21%, respectively. The intra- and inter-day accuracies of the standards ranged from 95.12-101.75% and 94.86-101.95%, respectively. The average recovery was calculated by (difference in marker compound levels in spiked and unspiked samples)/(amount of spiked standard) × 100. The percent recovery of each standard ranged from 98.06-101.71% and RSDs were less than 1.86% (see Table 3). These data verify that the developed method is highly reproducible. The standard solutions were also stable, maintaining 96.5% of their initial compound concentrations under all conditions tested.

Analysis and Evaluation of AR Samples. The developed HPLC/UV method was applied to the simultaneous quantification of three marker compounds in 86 samples of AR. Each sample was analysed in triplicate to ensure reproducibility. All three of the marker compounds were found in all samples but their relative concentrations varied widely. Levels of alisol C acetate, alisol B, and alisol B acetate were 0.07-0.45, 0.38-10.32, and 1.13-8.59 mg/g dried weight, respectively. The distribution of marker compounds also differed according to the origin of the sample (Table 4). More alisol B acetate was detected in Korean samples than in Chinese samples. Chinese samples, however, contained higher levels of alisol B. This result is consistent with a previous report stating that Korean AR contains higher levels of alisol B acetate than Chinese AR.[24] However, discrimination between Korean and Chinese samples was not possible

Table 4. Average contents and ranges of Alismatis Rhizoma samples

Marker compounds	Korean samples (n=53)	Chinese samples (n=33)	Total (n=86)
Alisol B	0.99 (0.38-3.18)	4.45 (2.01-10.32)	2.32 (0.38-10.32)
Alisol B acetate	3.08 (1.57-8.59)	1.97 (1.13-4.38)	2.65 (1.13-8.59)
Alisol C acetate	0.20 (0.07-0.45)	0.13 (0.08-0.43)	0.18 (0.07-0.45)

Unit: mg/g, range of content in parenthesis.

based solely on levels of alisol B acetate due to the wide natural variability. Levels of alisol C acetate were relatively low in all samples. Thus, alisol C acetate was deemed in appropriate as a marker compound for routine AR quality control. No written regulations exist regarding the types and levels of AR marker compounds in Korean, Chinese, and Japanese pharmacopoeias. The results of the current study suggest that regulatory levels for alisol B and its acetate in AR could be set at no less than 0.36 mg/g and 1.29 mg/g of dried sample, respectively, based on the "Criteria for Levels of Marker Compounds in Herbal Drugs" method.[30]

Pattern Recognition Analysis. The 86 AR samples all yielded similar chromatograms. The extent of the similarities made it difficult to distinguish the samples based on quantitative analyses of the marker compounds alone. However, subtle differences in peak intensity and/or area may have been the result of differences in cultivation area, harvesting season, climate, method of collection, washing, drying, preservation procedure, and storage conditions. These subtle differences may be exploited for sample differentiated by pattern analysis. The pattern recognition analysis for AR based on 11 common peaks was carried out as a preliminary try. However, not all peaks contributed to the classification of AR samples in different origin. Thus, the number of peaks

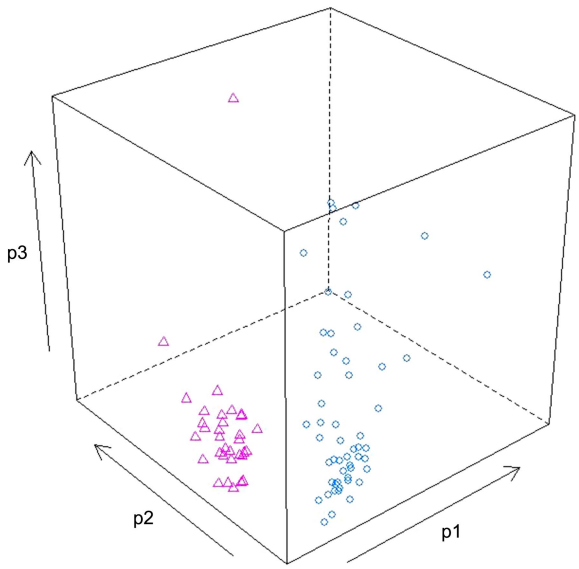

Figure 3. The PLS-DA analysis of Alismatis Rhizoma samples from Korea (circles) and China (triangles).

selected for pattern analysis was reduced to the number without changing the accuracy of the classification. Finally, three marker compounds were selected and showed good results for classification of Korean and Chinese samples. Pattern recognition analysis was performed with three discrimination models: LDA, PLS-DA, and KNN. All of the models yielded 100% differentiation between Korean and Chinese samples (Figure 3). Occasionally, an overfitting occurred in classification with small size of samples and showed unreasonable good results. The sample size of this experiment was relatively large, nonetheless, the possibility of the overfitting could not be fully excluded.

Conclusions

A simple, quantitative pattern recognition method based on three bioactive compounds, alisol B, alisol B acetate, and alisol C acetate, was developed and validated for the quality control of AR. The method was applied to analyse 86 AR samples from Korea and China. All three of the target compounds were found in all samples, but with a high degree of concentration variability. Levels of alisol B acetate were generally higher in Korean samples than in Chinese samples, while levels of alisol B were higher in Chinese samples. The results suggest regulatory levels of no less than 0.36 mg of alisol B and 1.29 mg of alisol B acetate per gram of dried AR. Pattern analyses based on discrimination models were remarkably accurate in differentiating Korean and Chinese samples.

Acknowledgments. This work was supported by the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815).

References

1. Korea Food and Drug Adminitration. *Korean Pharmacopoeia*, 7th ed.; Seoul, 2007.
2. Do, H. B.; Dang, Q. C.; Bui, X. C.; Nguyen, T. D.; Do, T. D.; Pham, V. H.; Vu, N. L.; Pham, D. M.; Pham, K. M.; Doan, T. N.; Nguyen, T.; Tran, T. *Plants and Animals for Medicines in Vietnam*, 1st ed.; Vietnamese Sci-tech Publisher: Hanoi, Vietnam, 2003, Vol II.
3. Bae, K. *Medicinal Plants of Korea*, 1st ed.; Kyohaksa: Seoul, Korea, 2000.
4. Murata, T.; Shinohara, M.; Miyamoto, M. *Chem. Pharm. Bull.* **1970**, *18*, 1369.
5. Fukuyama, Y.; Geng, P.; Wang, R.; Yamada, Y.; Nakagawa, K. *Planta Med.* **1998**, *54*, 445.
6. Yoshikawa, M.; Hatakeyama, S.; Tanaka, N.; Fukuda, Y.; Murakami, N.; Yamahara, J. *Chem. Pharm. Bull.* **1992**, *40*, 2582.
7. Yamaguchi, K.; Ida, Y.; Satoh, Y.; Nakajima, Y.; Shoji, Z. *Acta Crystallogr. Sect. C: Cryst. Struct. Commun.* **1994**, *50*, 738.
8. Yoshikawa, M.; Yamaguchi, S.; Matsuda, H.; Kohda, Y.; Ishikawa, H.; Tanaka, N.; Yamahara, J.; Murakami, N. *Chem. Pharm. Bull.* **1994**, *42*, 1813.
9. Matsuda, H.; Tomohiro, N.; Yoshikawa, M.; Kubo, M. *Biol. Pharm. Bull.* **1998**, *21*, 1317.
10. Lee, S. H.; Lee, Y. S.; Jung, S. H.; Shin, K. H.; Kim, B. K.; Kang, S. S. *Arch. Pharm. Res.* **2003**, *26*, 727.
11. Lee, S. M.; Kho, Y. H.; Min, B. S.; Kim, J. H.; Na, M. K.; Kang, S. J.; Maeng, H. Y.; Bae, K. H. *Arch. Pharm. Res.* **2001**, *24*, 524.
12. Kim, N. Y.; Kang, Y. H.; Pae, H. O.; Choi, B. M.; Chung, H. T.; Myung, S. W.; Song, Y. S.; Sohn, D. H.; Kim, Y. C. *Biol. Pharm. Bull.* **1999**, *22*, 1147.
13. Kubo, M.; Matsuda, H.; Tomohiro, N.; Yoshikawa, M. *Biol. Pharm. Bull.* **1997**, *20*, 511.
14. Huang, Y. T.; Huang, D. M.; Chueh, S. C.; Teng, C. M.; Guh, J. H. *Cancer Lett.* **2006**, *231*, 270.
15. Chang, Y. X.; Yan, D. M.; Chen, L. L.; Ding, X. P.; Qi, J.; Kang, L. Y.; Zhang, B. L.; Yu, B. Y. *Chem. Pharm. Bull.* **2009**, *57*, 586.
16. Chitlange, S. S.; Kulkarni, P. S.; Patil, D.; Patwardhan, B.; Nanda, R. K. *J. AOAC Int.* **2009**, *92*, 1016.
17. Wang, Y.; Han, T.; Zhang, X. G.; Zheng, C. J.; Rahman, K.; Qin, L. P. *Chromatographia* **2009**, *70*, 143.
18. Yang, L.; Xu, S. J.; Feng, Q. R.; Liu, H. P.; Tian, R. T.; Xie, P. S. *J. Liq. Chromatogr. R. T.* **2009**, *32*, 2893.
19. Zhang, Y. Y.; Dan, M.; Wu, J. B.; Yang, H. Z.; Huang, H.; Qi, Y.; Wei, S.; Okuyama, T.; Nakajima, K. *Chromatographia* **2008**, *68*, 101.
20. Ma, C. F.; Wang, H. H.; Lu, X. G.; Xu, W.; Liu, B. Y. *J. Chromatogr. A* **2008**, *1186*, 412.
21. VanMen, C.; Jang, Y. S.; Zhu, H. M.; Lee, J. H.; Trung, T. N.; Ngoc, T. M.; Kim, Y. H.; Kang, J. S. *Phytochem. Anal.* **2012**, *23*, 359.
22. Sim, C. O.; Ahmad, M. N.; Ismail, Z.; Othman, A. R.; Noor, N. A. M.; Zaihidee, E. M. *Sensors* **2003**, *3*, 458.
23. Men, C. V.; Jang, Y. S.; Lee, K. J.; Lee, J. H.; Quang, T. H.; Long, N. V.; Luong, H. V.; Kim, Y. H.; Kang, J. S. *Arch. Pharm. Res.* **2011**, *34*, 2065.
24. Lee, S. M.; Kang, J. S.; Hwang, G. S.; Kim, Y. H.; Lee, C. G.; Yeo, W. H.; Bae, K. *Arch. Pharm. Res.* **2004**, *27*, 460.
25. Park, J. C.; Hur, J. M.; Kim, S. E. *J. Kor. Soc. Food Sci. Nutr.* **2005**, *34*, 243.
26. Wen, H.; Li, W.; Peng, G.; Chi, Y. *Zhong Yao Cai* **1998**, *21*, 595.
27. Ahn, M. J.; Lee, C. H.; Shin, Y. W.; Chun, M. S.; Kim, C. Y.; Kim, J. W. *Nat. Prod. Sci.* **2008**, *14*, 152.
28. Murata, T.; Shinohara, M.; Hirata, T.; Kamiya, K.; Nishikawa, M.; Miyamoto, M. *Tetrahedron Lett.* **1968**, *9*.
29. Zhan, Z. J.; Bian, H. L.; Shan, W. G. *Acta Crystallogr. Sect. E Struct. Rep.* **2008**, *E64*, o2231.
30. Kang, J. S.; Jang, Y. S.; Baek, K. O. Method for the Determination of Criteria of Natural Products by Content Monitoring. KR patent 10-1123081. 2012.